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Investigations of Metals and Metal-Antibrowning Agents Effects on Polyphenol Oxidase Activity from Red Poppy Leaf

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***Abstract*—**Heavy metals are one of the major groups of contaminants in the environment and many of them are toxic even at very low concentration in plants and animals. However, some metals play important roles in the biological function of many enzymes in living organisms. Metals such as zinc, iron, and cooper are important for survival and activity of enzymes in plants, however heavy metals can inhibit enzyme which is responsible for defense system of plants. Polyphenol oxidase (PPO) is a copper-containing metalloenzyme which is responsible for enzymatic browning reaction of plants. Enzymatic browning is a major problem for the handling of vegetables and fruits in food industry. It can be increased and effected with many different futures such as metals in the nature and ground. In the present work, PPO was isolated and characterized from green leaves of red poppy plant (*Papaverr hoeas*). Then, the effect of some known antibrowning agents which can form complexes with metals and metals were investigated on the red poppy PPO activity. The results showed that glutathione was the most potent inhibitory effect on PPO activity. Cu(II) and Fe(II) metals increased the enzyme activities however, Sn(II) had the maximum inhibitory effect and Zn(II) and Pb(II) had no significant effect on the enzyme activity. In order to reduce the effect of heavy metals, the effects of metal-antibrowning agent complexes on the PPO activity were determined. EDTA and metal complexes had no significant effect on the enzyme. L-ascorbic acid and metal complexes decreased but L-ascorbic acid-Cu(II)-complex had no effect. Glutathione–metal complexes had the best inhibitory effect on Red poppy leaf PPO activity.

***Keywords*—**Inhibition, metal, red poppy, Polyphenol oxidase (PPO).

I. Introduction

HEAVY metals are natural components of Earth and cannot be degraded or destroyed. To a small extent they enter human and animal bodies via food, drinking water and air. As trace elements, some heavy metals (e.g. copper, nickel, selenium, zinc) are essential to maintain the metabolism of the plants and animals. However, at higher concentrations they can lead to poisoning of environment and organisms. Heavy metal poisoning is a major environmental problem that can reduce both the productivity and safety of plant products as foods [4]-[6]. These heavy metals affect the enzymes at the cellular level by altering enzyme activities, resulting in enzymes activation or inhibition in living organisms [11]. Several investigations were carried out to assess the effects of different metals (singly or in combination) on various plants in relation to their biochemical response [9], [18].

Metals also play important roles in the biological functions of many enzymes in living organisms. The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators. They participate directly in the catalytic mechanism. Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing metalloenzyme, widely distributed in plants and microorganisms. Polyphenol oxidase (PPO) is responsible for the enzymatic browning reaction during the handling, storage and processing of fruit and vegetables. In the presence of molecular oxygen, PPO catalyzes the o-hydroxylation of monofenols to o-diphenols (monophenolase activity) and oxidation of the o-diphenols to o-quinones (diphenolase activity) [14]. The quinones are then polymerized to brown or black pigments which lead to nutritional modifications and reduce food quality. This has been a serious problem in the food industry. Several studies have focused on the inhibition of enzymatic browning by ascorbic acid. Ascorbic acid can reduce o-quinones, produced by PPO-catalyzed oxidation of polyphenols, back to dihydroxy polyphenols and has been widely used as an antibrowning agent for processing of fruits and vegetables. However, the effect of ascorbic acid is temporary since once it is added, it is completely oxidized and o-quinones could accumulate, leading to browning pigment formation [7], [23]. Sulphydryl (thiol) compounds L-cysteine and reduced glutathione (GSH) are also excellent inhibitors of browning of potato powder [14].

Extensive studies have been carried out on the purification, characterization and inhibitors of PPO enzyme. Polyphenol oxidases have been purified and characterized from various plant sources such as eggplant [1], peppermint [19], artichoke [3], broccoli [16], butter lettuce [17], medlar fruits [12], iceberg lettuce [10], mulberry [2], musa paradisiacal leaf [26] and spider flower [15].Some of the natural agents proposed to have an inhibitory effect on PPO are honey, natural aliphatic alcohols, L-ascorbic acid (vitamin C), L-cysteine. It has been suggested that the amino acid, cysteine, can form a stable complex with copper, thus retarding enzymatic browning [24]. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in polyphenol oxidase reactions. It has been used to reduce enzymatic browning during food process [14]. EDTA is generally used in combination with other chemical treatments for the prevention of enzymatic

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browning in foods. It is not very effective as an inhibitor of peach polyphenol oxidase [27]. These anti-browning agents inhibit PPO enzyme and lower the browning reaction during the process. Some heavy metals in environment also inhibit PPO activity and reduce the enzyme activity during the growth of plants. To reduce the effect of the heavy metals on enzymes in the plant, many studies regarding interactions between chemicals, metals and various substances with different parameters of enzymes have gained considerable interest [6]. Although there are studies regarding characterization and purification of PPO from various plants, no reports have been found on purification and characterization of PPO enzyme from green leaves of red poppy (*Papaverr hoeas*) plant. Therefore, the objective of our study was focused in two parts, first we isolated and characterized the PPO enzyme from green leaves of red poppy (*Papaverr hoeas*). Second, we investigated how heavy metals, anti-browning agents and both of their complexes could affect the poppy leaf PPO activity.

II. Material and Methods

A. Chemicals

Green leaves of red poppy (*Papaverr hoeas*) used in this study was obtained from Sakarya region and stored at –20°C until used. Polyviniyl polypyrolidone (PVP), Sephadex G-100, $(NH_4)_2SO_4$ and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

B. Extraction and Purification

15 g of green leaves of red poppy (*Papaverr hoeas*) was obtained from local Sakarya region. After that samples were added to 10 ml 50mM sodium phosphate buffer (pH; 7.0), 0.3 g polyvinylpolypyrolidone (PVPP), and extraction was prepared. The mixture was homogenized with blender. After the filtrate was centrifuged at 14.000xg for 30 min and supernatant was collected. Extraction was fractionated with $(NH_4)_2SO_4$, solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% saturation. The mixture was centrifuged at 14,000xg for 30 minutes and the precipitate was dissolved in a small amount of phosphate buffer and then dialyzed at 4°C in the same buffer for 24 h with three changes of the buffer during dialysis. The dialyzed enzyme extract was centrifuged and loaded onto Sephadex G-100 column previously equilibrated with extraction buffer, and washed with the same buffer to remove unbound proteins. The eluate was used as the PPO enzyme source in the following experiments. The amount of PPO was performed according to method of Bradford with bovine serum albumin as standard [8].

C. Enzyme Assay

PPO activity was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm. PPO activity was assayed using catechol, 4-methyl catechol, caffeic acid and pyrogallol as substrates. The optimum pH, temperature obtained for all substrates was used for determining substrate specificities. For each substrate, the kinetic data were plotted as reciprocals of activities versus substrate concentrations. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined as the reciprocal absolute values of the intercepts on the x- and y-axes, respectively, of the linear regression curve. Substrate specificity (Vmax, Km) was calculated by using the data obtained on a Lineweaver-Burk plot [21].

D. Effect of pH and Temperature

PPO activity, as a function of pH, was determined in a pH range of 4.5–5.5 in 50 mM acetate buffer, 6.5–7.5 in 50 mM phosphate buffer and 8.5–9.5 in 50 mMTris–HCl and Tris-Base buffer. The optimum pH values obtained from this assay were used in all the other experiments. The effect of temperature on PPO activity obtained at different temperature values (4-60ºC) and the optimum temperature of poppy PPO was determined.

E. Effect of Metals and Antibrowning Agents

Cu(II) ($CuSO_4$) , Fe(III) ($FeCl_3$), Zn(II) ($ZnSO_4$), Pb (II) ($PbCl_2$), Sn(II) ($SnCl_2$), Hg (II) ($HgCl_2$), Ca (II) ($CaCl_2$), Mg (II) ($MgCl_2$), Na (I) (NaCl), Co (II) ($CoCl_2$), as metals were used to determine their effects on poppy PPO enzyme activities at their different concentrations (1 and 10 mM).

L-cysteine, glutathione (GSH), ethylene diamine tetraacetic acid (EDTA) and L-ascorbic acid as anti-browning agents were used to determine their effects on the PPO enzyme activities at their different concentrations (1 and 10 mM).

Each metal and each anti-browning agent were pre-incubated separately to form metals-anti browning agent complexes for 30 minutes at 25ºC. Then, the effects of complexes on the PPO enzyme activities were determined by kinetic methods using catechol as a substrate at pH 7.0. The remaining enzyme activities were calculated for all the metals and metal-anti-browning agent complexes.

III. Results and Discussion

A. Purification of Poppy PPO

A sequential purification of poppy PPO, starting from crude extract from poppy leaves, precipitation with $(NH_4)_2SO_4$ from 10% to 80% salt saturation, dialysis, to gel filtration chromatography, gave purified poppy PPO. Results for the purification of PPO were given in Table I. Ammonium sulphate fractionation as the first step of poppy PPO purification proved convenient and effective to remove large amounts of non-targeted proteins and brown pigments. When the purification steps were examined, there were 1.44 fold purifications after ammonium sulfate precipitation. Further purification was followed by loading the dialyzed enzyme on to the Sephadex G-100 gel filtration column. After, the gel filtration chromatography, the most active fractions were collected and examined for characterization and purification. The purification procedure gave an overall purification of 4.2 fold, and the purified poppy PPO had an overall activity yield of 19.21% (Table I).

TABLE I
STEPS FOR PURIFICATION OF POLYPHENOL OXIDASE FROM POPPY LEAF

Puricifation steps	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	51650	4278	12.07	1	100
Ammonium sulfate	23320	1343	17.36	1.44	45
Sephadex G-100	9920	195	50.87	4.21	19.21

B. Effect of pH and Temperature

Optimal pH and temperature of the PPO were estimated by using four different substrates (Table II). Optimum pH value for poppy PPO was found to be 7.0 with catechol and 4-methyl catechol, 8.0 with pyrogallol and 5.5 with caffeic acid. The pH optimum for enzyme-catalyzed oxidation of catechol in phosphate buffer was found to occur at pH 7.0 (Fig. 1). The pH optimum for poppy PPO is within the range (pH4.0–7.0) in which most PPOs display optimum activity [13], [16], [22]. The low activity observed at more acidic and basic pH values may be due to enzyme instability at these pH values (Fig. 1).

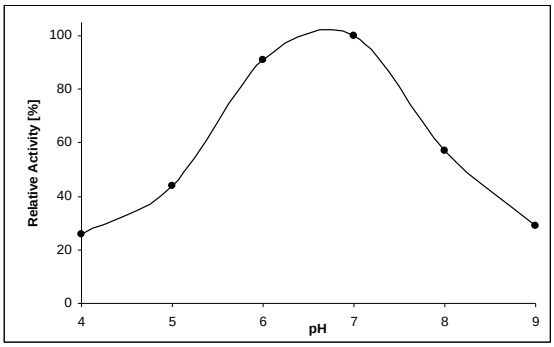


Fig. 1 Effect of pH on poppy PPO activity

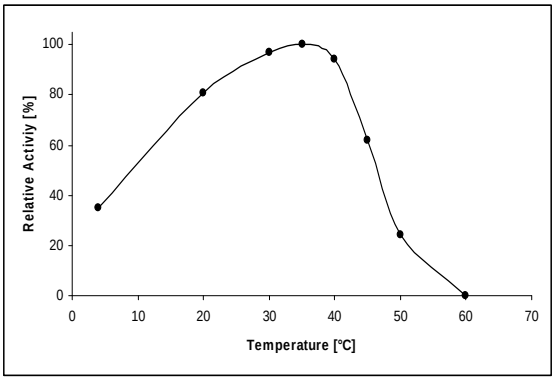


Fig. 2 Effect of temperature on poppy PPO activity

The enzyme showed the highest activity at 35°C with the substrate catechol. The activity of PPO was also measured at different temperatures at optimum pH (Table II). The enzyme showed the highest activity at 35°C with the substrate catechol and 4-methyl catechol, at 35°C with pyrogallol and 25°C with caffeic acid. This value was similar to those of peppermint PPO [19] and medlar PPO [13]. But the temperature optimum values were different from Barbados cherry PPO [20].

TABLE II
OPTIMUM ACTIVITY CONDITIONS AND SUBSTRATE SPECIFICITIES OF POPPY PPO

Substrates	Km (mM)	Vmax (μM/min)	Vmax/Km (min^{-1})	Optimum pH	Optimum temperature (°C)
Catechol	7,32	0.0024		7.0	35
4-Methyl Catechol	1,87	$1,69.10^{-3}$		7.0	35
Pyrogallol	1.35	9.1	6.7	8.0	35
Caffeic Acid	7.1	13.1	1.8	5.5	25

C. Substrate Specificity

Km and Vmax values of poppy PPO were calculated from the Lineweaver–Burk graphs and the results are shown in Table II. All the substrates, namely catechol, 4-methyl catechol, pyrogallol, and caffeic acid, were oxidized significantly by the enzyme, displaying simple Michaelis–Menton kinetics. Linear regression analysis of v versus S determined Vmax and Km values for each substrate (Table II). The highest Km values were shown by caffeic acid, 4-methyl catechol and catechol and the lowest by pyrogallol. In order to evaluate the substrate specificity, Vmax/Km ratio was taken It appears that the substrate-binding site of poppy PPO has a high affinity for small o-diphenols, such as catechol and 4-methyl catechol and less affinity for the larger o-diphenols, caffeic acid, and triphenol-pyrogallol. This result was consistent with the previous report on plant PPOs [20].

D. Effect of Various Metals, Antibrowning Agents and Their Complexes

Effects of various metals and other chemical reagents on the enzyme activity were studied by using catechol as the substrate (Table III). It is clear that glutathione (GSH) was found to be the most potent anti-browning agent followed by L-ascorbic acid, L-cysteine and EDTA at 10mM concentration (Table III). Among the anti-browning agents, L-Cysteine was reported to be a strong inhibitor of apple PPO [25] and ascorbic acid is effective inhibitor for different PPOs [2], [7], [10]. Since cysteine and ascorbic acid are naturally occurring substances and non-toxic, they may be useful for preventing the enzymatic browning of poppy. L-cysteine and reduced glutathione which are reactive thiol compounds are also excellent inhibitors of browning of plants PPO [13], [14]. One of the effective anti-browning agent is EDTA which is a known chelating agent permitted for use in the food industry as a chemical preservative. EDTA showed minimum inhibition to poppy PPO activity like cherry PPO [20].

The effect of Cu(II), Fe(III), Zn(II), Sn(II), Hg (II), Pb (II), Ca (II), Mg (II), Na (I), Co (II), as metals were determined on poppy PPO enzyme activity at different concentrations (1-10 mM) (Table III). The results showed that Cu(II) and Fe(II) metals increased the enzyme activity at 10 mM. Similar activation of artichoke heads PPO activity by Cu(II) and Fe(II) were reported by Aydemir in the same concentrations [3]. However, Sn(II) had the maximum inhibitory effect on the enzyme activity at 10 mM. Although heavy metals are toxic

for all plant and human health, Zn(II), Hg (II) and Pb (II) had no significant effect on the enzyme activities, whereas the same metals had heavily inhibitor effect on the other plant PPOs [15].

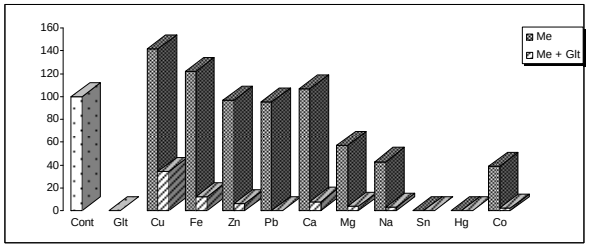
The effects of metal and anti-browning agent complexes were also tested on poppy PPO enzyme activity. The results are in Fig. 3. The results showed that, EDTA and metal complexes had no significant effect on the enzyme activity. L-ascorbic acid and metal complexes decreased the enzyme activities but L-ascorbic acid-Cu(II)-complex had no effect on the enzyme activity. L-Cys-Pb, L-Cys-Hg and L-Cys-Sn had inhibitory effect respectively but the other L-Cys-metal complexes had no effect on the enzyme activities. GSH–metal complexes had the best inhibitory effect on the enzyme activities except GSH-Fe complex. The results suggested that the metal-anti browning agent complexes may increase the inhibitory effect of anti-browning agents for the enzymatic browning in plants and reduce the effect of heavy metals on the enzyme activity.

TABLE III
THE EFFECTS OF METAL AND METAL-ANTI-BROWNING AGENTS ON POPPY PPO ENZYME ACTIVITY

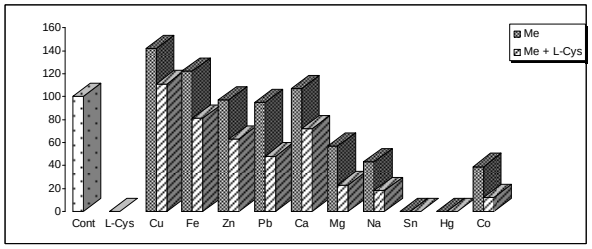
Metals	Remaining Activity (%) (1 mM)	(10 mM)
$CuSO_4$	107	142
$FeCl_3$	115	122
$ZnSO_4$	102	97
$PbCl_2$	100	95
$CaCl_2$	100	107
$MgCl_2$	82	57
NaCl	68	43
$SnCl_2$	13	0
$HgCl_2$	33	0
$CoCl_2$	62	39
Anti-Browning Agents		
EDTA	88	82
L- Ascorbic Acid	43	0
Glutathione (GSH)	0	0
L-Cysteine	63	42

IV. CONCLUSION

There were two purpose of this work, one was to purify and characterize polyphenol oxidase (PPO) from a new plant source which is red poppy (*Papaverr hoeas*). Second was to reduce the effect of heavy metals and increase the effect of anti-browning agent on this new plant PPO enzyme. The results of this work showed that the PPO enzyme was partially purified and characterized from poppy. The data suggested that the metal-anti browning agent complexes may reduce the effect of toxic heavy metals on the enzyme activity and increase the inhibitory effect of anti-browning agents for the enzymatic browning in plants. Browning in fruits and vegetables is recognized as a serious problem in the food industry. Further studies are warranted to understand the PPO inhibitor in relation to the browning reaction of plant during storage and processing.



(a)



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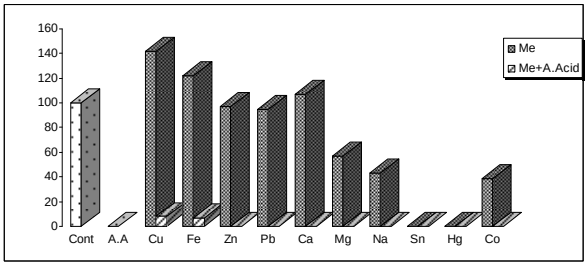
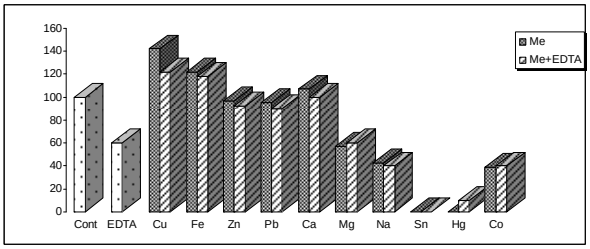


(c)



(d)

Fig. 3 Relative activity (%) profiles for poppy PPO against metal-anti-browning agent complexes. (a) Metals and Metal+GSH, (b) Metal and Metal+L-Cystein, (c) Metal and Metal+ Ascorbic Acid, (d) Metal and Metal+ EDTA

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